Free Radical Research, March 2005; 39(3): 325–336

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Aromatic and aliphatic mono- and bis-nitroxides: A study on their radical scavenging abilities

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Accepted by Professor E. Niki

(Received 13 October 2004; in revised form 4 December 2004)

Abstract
Nitroxide radicals are an emerging class of interesting compounds with versatile antioxidant and radioprotective properties. All literature studies have so far concentrated on compounds bearing only one nitroxide function. Here, we now investigate and compare the radical scavenging behaviour and antioxidant activity of aromatic indolinonic and aliphatic piperidine bis-nitroxides, i.e compounds bearing two nitroxide functions. Their corresponding mono-derivatives were also studied for comparison. Radical scavenging activity was investigated using EPR and UV–Vis spectroscopy by following spectral changes in acetonitrile of the nitroxides in the presence of alkyl and peroxyl radicals generated, respectively, under anoxic or aerobic conditions from thermal decomposition of AMVN [2,2′-azobis(2,4-di-methylvaleronitrile)]. Antioxidant activity of the nitroxides was evaluated by monitoring conjugated dienes (CD) formation during methyl linoleate micelles peroxidation and by measuring carbonyl content in oxidized bovine serum albumin (BSA). The results show that: (a) each nitroxide moiety in bis-nitroxides scavenges radicals independent of each other; (b) aliphatic nitroxides do not scavenge peroxyl radicals, at least under the experimental conditions used here, whereas indolinonic aromatic ones do: their stoichiometric number is 1.14 and 2.17, respectively, for mono- and bis-derivatives; (c) bis-nitroxides are roughly twice more efficient at inhibiting lipid peroxidation compared to their corresponding mono-derivatives. Although this study provides only comparative information on the relative radical-scavenging abilities of mono- and bis-nitroxides, it helps in understanding further the interesting reactivity of these compounds especially with regards to peroxyl radicals where many controversies in the literature exist.

Keywords: *Mono- and bis-nitroxides, antioxidants, peroxyl radicals, alkyl radicals, EPR and UV–Vis spectroscopy*

Introduction

The increasing knowledge on the multiple aspects of oxidative stress, principally in terms of health care, has led to an escalation in research for ways of approaching this problem. From this point of view, the use of nitroxide radicals as antioxidants and as radioprotectors may be of some importance since their protective effects in a multiplicity of biological systems at the molecular, cell, organ, and whole-body levels against oxidative stress, have been widely established [1–5]. Nevertheless, all the antioxidant studies which have so far appeared in the literature on these compounds, including those carried out by our research group [6–10], have focused on mono-nitroxides. At this stage, we thought that it would be interesting to take into account compounds bearing two $>N—O\cdot$ functional groups in the same molecule, i.e. bis-nitroxide radicals, and to study their radical-scavenging behaviour and antioxidant activity. For this purpose, two different groups of nitroxides were chosen, aromatic indolinonic and aliphatic piperidine nitroxides, because their radical scavenging abilities towards radical species differ, in most cases, as a result of their

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2005 Taylor & Francis Ltd
DOI: 10.1080/10715760400028019

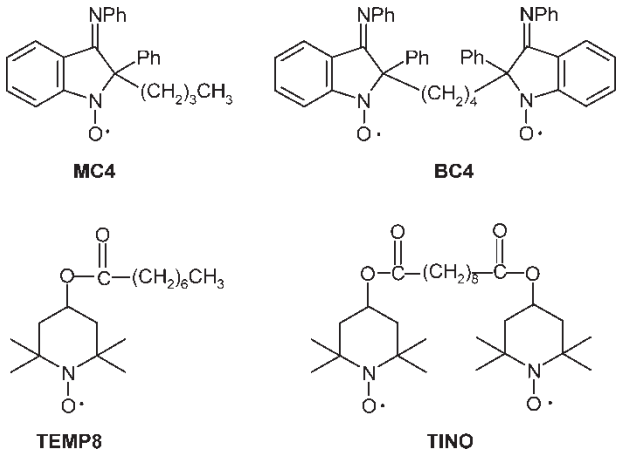


Figure 1. Chemical structures of compounds studied.

chemical structure. In fact, while the reactivity of the former group with a wide range of radicals (alkyl, alkoxyl, peroxyl, nitric oxide, aminyl, superoxide, hydroxyl, thiyl) [11–16] is amply documented, reactions of aliphatic nitroxides with radicals is only clear in the case of alkyl [17] and superoxide radicals for which a SOD-mimetic activity has been attributed [18,19], whereas with peroxyls, controversies still exist. As a representative of the aromatic nitroxides, two indolinonic, mono- (MC4) and bis- (BC4) nitroxides each bearing a butyl side chain in position 2 were synthesised, while for the aliphatic ones, a piperidine nitroxide bearing an octyl side chain was prepared (TEMP8) for analogy with its bis-nitroxide (TINO) bearing a similar side chain (Figure 1). The nitroxides were reacted with peroxyl and alkyl radicals generated from thermal decomposition of AMVN [2,2′-azo-bis(2,4-di-methylvaleronitrile)], a lipid soluble radical generator, according to whether the reactions were carried out under aerobic or anoxic conditions, respectively, and the reactions were followed using EPR and UV–Vis spectroscopy. Antioxidant activity was also evaluated in model systems by monitoring methyl linoleate micelles peroxidation and by measuring the content of carbonyl groups in oxidized bovine serum albumin (BSA) in the presence of nitroxides.

Materials and methods

Materials

The indolinonic nitroxides MC4 (1,2-dihydro-2-butyl-2-phenyl-3*H*-indole-3-phenylimino-1-oxyl and BC4 [1,4-di-(1-oxyl-2-phenyl-3-phenylimino-3*H*-indol-2-yl)-butane] (Figure 1) were synthesized according to the method previously described with minor modifications, by reacting the appropriate Grignard reagent with 2-phenyl-3-phenylimino-3*H*-indole-1- oxide [20]. 1,4-Dibromobutane [$Br(CH_2)_4Br$] was used for their preparation. The corresponding di-magnesium bromide was prepared as follows: the dibromoalkane (50 mmoles) in 10 ml tetrahydrofuran (THF) was added dropwise under argon to a suspension of magnesium (50 mmoles) in 10 ml THF containing traces of I_2. When one third of the dibromoalkane was added, the mixture was gently heated to initiate the reaction and after addition of the remaining dibromoalkane, the mixture was refluxed for 10 min. The Grignard reagent solution (approximate yield 70%) was added dropwise to 2-phenyl-3-phenylimino-3*H*-indole-1-oxide (6.8 mmoles) dissolved in 30 ml THF. The mixture was left to react for 1 h under magnetic stirring and under argon atmosphere. The reaction solution was then poured into an aqueous solution of 5% NH_4Cl and extracted with diethyl ether (3 × 40 ml). The organic layer was dried over Na_2SO_4 and concentrated to a small volume; PbO_2 (50 mmoles) was then added and left to react under magnetic stirring for 2 h. After filtration and concentration, the nitroxides were purified by silica gel column chromatography eluting with cyclohexane/ethylacetate 9:1. The mono-nitroxide (first red spot) was the first product to be isolated, followed by the bis-nitroxide (second red spot). The identity and purity of the compounds were checked by thin-layer chromatography, by mass spectroscopy on a Carlo Erba QMD 1000 spectrometer (Milan, Italy) in EI^+ mode and by electron spin resonance spectroscopy on a Bruker EMX EPR spectrometer (Bruker, Karlsruhe, Germany) equipped with an XL Microwave frequency counter, Model 3120 for the determination of the *g*-factors. The EPR spectra of the nitroxides recorded in chloroform were simulated on the basis of the following hyperfine coupling constants in Gauss: nitroxide MC4, $a_N(NO\cdot) = 9.73$, $a_H(H5) = 3.35$, $a_H(H7) = 3.17$, $a_H(H4) = 1.12$, $a_H(H6) = 1.06$, $a_H(1H, C2) = 0.37, a_H(1H, C2) = 0.17, a_N(1N, C3) = 0.78$ (g-factor $= 2.0056_4$). In the case of the bis-nitroxide BC4, we were unable to record well-resolved spectra due to the mutual interaction of the two nitroxide groups present in the same molecule that results in signal broadening. Only the hyperfine coupling constants for the nitrogen of this group could be assessed. The nitrogen shows $a_N = 9.73$, whilst the g-factor for BC4 is 2.0062_1. The mass spectra showed the following: MC4, calculated for $C_{24}H_{23}N_2O$, 355.46, found: m/e (relative intensity), 355 (M^+, 20), 339 (28), 299 (19), 282 (18), 211 (100); BC4, calculated for $C_{44}H_{36}N_4O_2$, 652.80, found: m/e (relative intensity), 652 (M^+, 1), 284 (40), 77 (65). The piperidine nitroxide TEMP8 [4-(octanoyl)-2,2,6,6-tetramethyl-piperidine-1-oxyl] was synthesized according to the method previously described by Rosantsev, by reacting 4-hydroxy-TEMPO with the appropriate octyl-chloride and triethylamine [21]. 4-Hydroxy-TEMPO (0.5 mmoles) was reacted with 2 mmoles of chloride and 2 mmoles of triethylamine in 20 ml toluene under magnetic stirring and at room temperature. After 1 h the reaction was complete. The reaction mixture was washed several times with

distilled water, extracted with 40 ml dichloromethane, dried over anhydrous sodium sulphate and concentrated. The nitroxide was a pale orange oil and used as obtained. The identity and purity of the compound was checked as for the indolinonic nitroxides. The EPR spectra recorded in chloroform showed the three typical bands of the nitrogen with $a_N(NO\cdot) = 15.83$, g-factor $= 2.0061_0$. The mass spectrum showed the following: Calculated for $C_{17}H_{32}NO_3$, 298.45, found: m/e (relative intensity), 298 (M^+, 42), 284 (15). Bis(2,2,6,6-tetramethyl-1-oxyl-piperidine-4-yl)sebacate (TINO) was a gift from Ciba Specialty Chemicals (Basel, Switzerland). Bovine serum albumin (Fraction V, A-6003), methyl linoleate (L-1643) as well as all other reagents and solvents were purchased from Sigma-Aldrich Chemical Co. (Milan, Italy). The lipid soluble, free-radical generator, AMVN [2,2′-azobis(2,4-dimethylvaleronitrile)], was kindly prepared and donated by Prof. R. Leardini from the University of Bologna (Italy) according to the method described in the literature [22].

UV–Vis spectroscopy experiments

UV–Vis spectroscopy was employed to follow the UV spectral changes of the nitroxides in the presence of AMVN. In a final volume of 3 ml, the UV spectra of 50 μM nitroxide and 6 mM AMVN in acetonitrile were recorded every 6 min for almost 1 h at 40°C on a UV Kontron 941 spectrophotometer, thermostatted at 40°C. When anoxic conditions were desired, the quartz cuvettes containing the samples were first sealed with rubber stoppers and purged thoroughly with nitrogen gas for 10 min using appropriate needles, after which they were transferred to the spectrophotometer for UV recordings.

EPR spectroscopy experiments

For the EPR study on the scavenging ability of the nitroxides toward radicals generated from AMVN, 200 μM nitroxide and 24 mM AMVN in acetonitrile in a final volume of 1 ml were transferred to glass capillary tubes (1 mm i.d.), thoroughly degassed under argon and then transferred to the EPR cavity for spectral measurements at 40°C until complete disappearance of the EPR signal. These were recorded on a Bruker EMX EPR spectrometer as mentioned previously, equipped with a Stelar VTC87 temperature controller. The spectra were recorded with the following instrumental settings: 5 mW microwave power, 0.5 G modulation amplitude and 100 kHz field modulation. When aerobic conditions were desired, the above mentioned solution was not subjected to degassing but transferred directly to the EPR cavity.

Peroxidation of methyl linoleate micelles

Methyl linoleate micelles peroxidation was monitored by measuring conjugated dienes (CD) at 234 nm. The micelles were prepared by mixing appropriate amounts in the following order, of methyl linoleate (1.5 mM), nitroxide from an acetonitrile stock solution (1 μM) and AAPH [2,2′-azobis(2-amidinopropane) dihydrochloride] (1 mM) which is a water soluble, free-radical generator with a buffer solution (5 mM phosphate, 0.1 mM EDTA, 0.9% NaCl, pH 7.4) of 0.5 M SDS (sodium dodecyl sulphate), in a final volume of 2 ml, followed by vigorous mixing for 1 min, according to a modified method of Foti et al. [23] Samples were then transferred to quartz cuvettes and placed in the sample compartment of the spectrophotometer thermostatted at 40°C. The progress of peroxidation was then monitored by recording the absorbance at 234 nm due to conjugated diene formation, for 2 h against an appropriate blank which lacked methyl linoleate.

The inhibition time (T_{inh}) was estimated as the point of intersection between the tangents to the inhibition- and propagation-phase curves, under precise oxidation conditions (see inset of Figure 8). The rate of radical generation from AAPH (R_i) in our experimental system was measured using Trolox, a water-soluble Vitamin E analogue, as the radical scavenger [24].

Protein oxidation

The protein samples were prepared by dissolving 3 mg/ml of BSA in 50 mM potassium phosphate buffer, 0.1 mM EDTA, pH 7.4. Samples were then incubated at 50°C for 1 h in the presence or absence of 5 mM AAPH and different final concentrations of nitroxides (50 and 100 μM). The nitroxides were added to the protein as acetonitrile solutions (2.5% v/v) and the mixture was vortexed prior to addition of AAPH for thorough incorporation. The degree of protein oxidation was monitored by the method of Levine et al. which uses the reaction of 2,4-dinitrophenylhydrazine (DNPH) with the carbonyl groups of oxidized proteins [25]. Briefly, 0.5 ml of 20 mM DNPH in 2.5 M HCl was added to 0.5 ml of each sample; blank samples lacked DNPH. Following 1 h of incubation at room temperature with continuous shaking, the protein was precipitated by addition of 2 ml 20% TCA and centrifuged at 3000*g* for 10 min. The protein was washed twice with ethanol:ethylacetate (1:1) and dissolved in 1 ml of 6 M guanidine HCl, pH 6.5. Protein carbonyls were then read at 370 nm and evaluated using a molar absorption coefficient of 22,000 $M^{-1}\,cm^{-1}$.

Appropriate controls were carried out throughout all the experiments described above and the results reported are an average of at least three independent experiments. The EPR and UV–Vis spectroscopy

results reported in the figures as well as the data reported in Figure 8 are representative of at least three experimental runs. Statistical comparisons were performed using the Student *t*-test and differences were regarded as statistically significant when *p* values were <0.05 (*), <0.01 (**).

Results

The use of azo-initiators to generate radicals in either a hydrophobic or hydrophilic milieu for studies of antioxidant activity and lipid peroxidation is a common practice and much literature exists testifying the success of these compounds [26–28]. These decompose unimolecularly at a temperature controlled rate without enzymes or biotransformation to give molecular nitrogen and two carbon-centred radicals. If oxygen is present in the system, then the latter react rapidly with oxygen to give peroxyl radicals. Therefore, by excluding or including oxygen in a system under investigation, one is capable of studying the reactivity of potential free radical scavengers toward alkyl radicals or peroxyl radicals, respectively.

For measuring the radical scavenging activity of mono- and bis-nitroxides toward alkyl and peroxyl radicals, in this study we used AMVN which is a hydrophobic radical initiator. The fate of the nitroxide radical was then followed using UV–Vis spectroscopy and EPR spectroscopy in homogeneous acetonitrile solution.

Figure 2A shows the UV spectral changes of MC4 in the presence of AMVN and in the absence of oxygen. The characteristic peak of the nitroxide at 290 nm disappears, the peak at 360 nm increases whilst more characteristically, a new peak at 260 nm appears. No more spectral changes were observed after 30 min incubation indicating that all the nitroxide present had been transformed. From the resulting spectrum obtained, we deduce that MC4 is transformed into the corresponding alkoxyamine. This is based on the well-known fact that nitroxides react at an almost diffusion controlled rate with carbon-centred radicals ($10^9\,M^{-1}s^{-1}$) by coupling at the nitroxide function to give this non-paramagnetic species as reported in Scheme 1 [11,12,17]. When BC4 is reacted with AMVN in the absence of oxygen the same spectral pattern as its corresponding mono-nitroxide is observed since the two compounds are practically the same and react in a similar fashion with alkyl radicals to give their corresponding alkoxyamines. However, in this case it took roughly 1 h for complete transformation of the bis-nitroxide (results not shown).

The spectral changes observed upon reaction of AMVN with the aliphatic nitroxides in anoxic conditions were not as remarkable as those obtained with the aromatic nitroxides. Nevertheless, a red shift

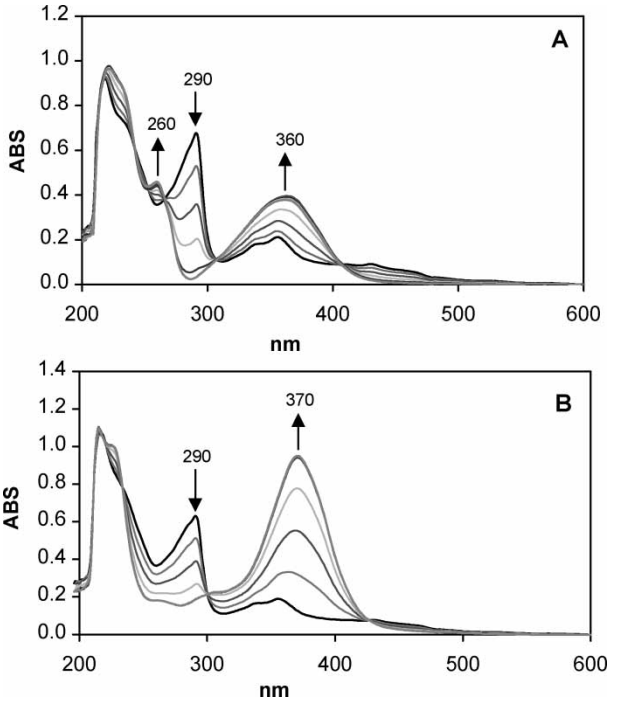
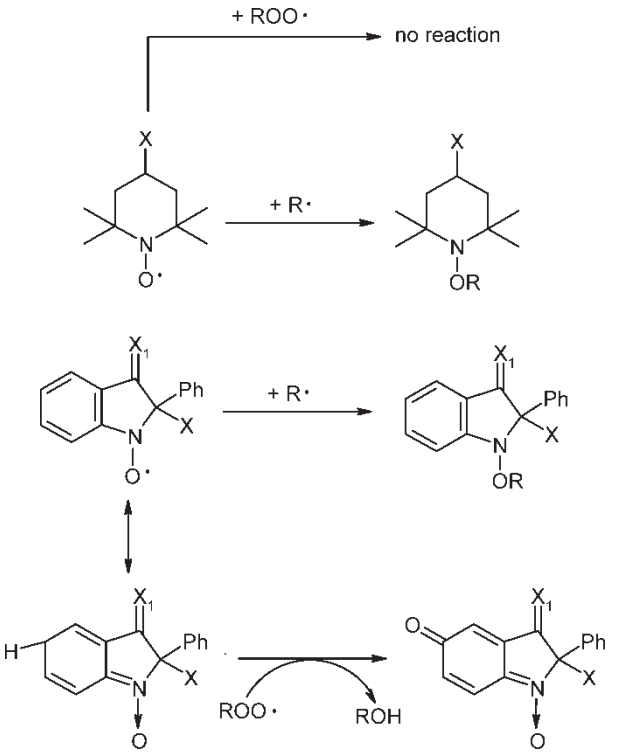


Figure 2. UV spectral changes of nitroxide MC4 in the presence of AMVN and in the absence (A) or presence of oxygen (B). The nitroxide (50 μM) was incubated with 6 mM AMVN in acetonitrile at 40°C for 1 h and recordings were taken every 6 min.



Scheme 1. Scheme showing the scavenging of alkyl (R·) and peroxyl radicals (ROO·) by aromatic and aliphatic nitroxides.

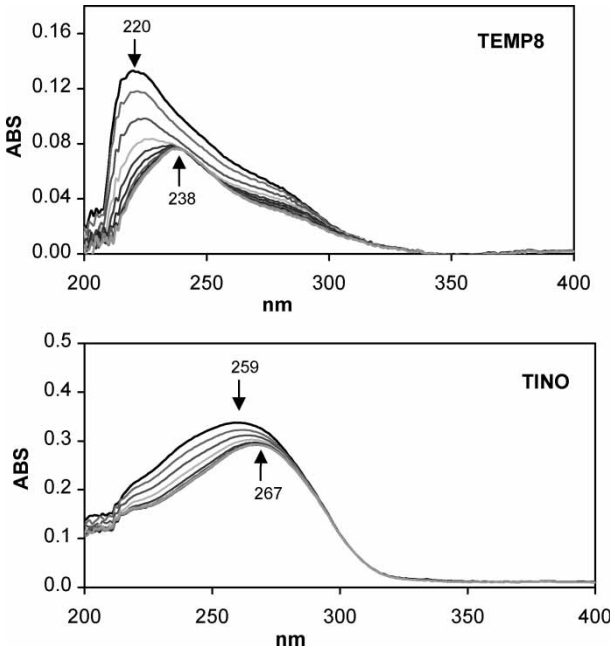


Figure 3. UV spectral changes of nitroxide TEMP8 and TINO in the presence of AMVN and in the absence of oxygen. The nitroxides (50 μM) were incubated with 6 mM AMVN in acetonitrile at 40°C for 1 h and recordings were taken every 6 min.

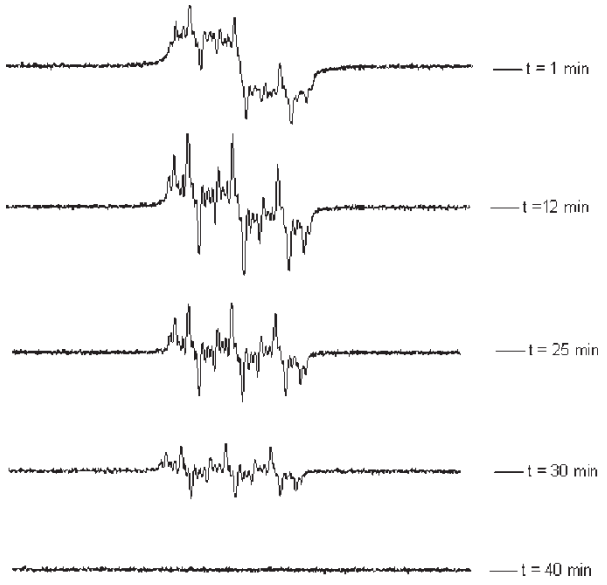


Figure 4. EPR spectral changes of nitroxide BC4 in the presence of AMVN and in the absence of oxygen. The nitroxide (200 μM) was incubated with 24 mM AMVN in acetonitrile at 40°C and recordings were continuously taken until signal disappearance.

together with a decrease in intensity of the maximum peak was observed with both TEMP8 and TINO. As can be observed in Figure 3, the maximum peak of TEMP8 shifts from 220 to 238 nm together with a decrease in absorbance and no more spectral changes were observed after 30 min incubation. Similarly, the same was observed for TINO with a shift from 259 to 267 nm. These changes are with all certainty ascribable to the formation of the corresponding alkoxyamine as described above for the aromatic nitroxides [17].

The results reported above were further confirmed by EPR experiments. Figure 4 shows the EPR spectra at different time points of BC4 with AMVN in anoxic conditions, therefore in the presence of carbon-centred radicals. At the beginning of the experiment the EPR spectrum is not well-resolved due to the mutual interaction of the two nitroxide groups present in the same molecule that results in signal broadening. However, as the time course of the experiment proceeds, the spectrum becomes resolved and its intensity increases after which it remains resolved but the intensity starts to decrease until complete disappearance after 40 min. This behaviour is explained by the fact that as the alkyl radicals are generated by AMVN, these react first with one of the nitroxide moieties of the molecule to give the alkylated hydroxylamine, an EPR-silent species, while the other $>N—O\cdot$ group is still unreacted. This leads to spectral resolution because there is no more interaction between the two paramagnetic moieties of the $>N—O\cdot$ groups; in fact the spectra obtained at 12 min is exactly the same as that of MC4. As time proceeds, the second $>N—O\cdot$ group also starts to react with the alkyl radicals leading to a decrease in intensity of the overall EPR signal up to its complete disappearance. This interesting behaviour was further observed with the aliphatic bis-nitroxide TINO. At the start of incubation, the EPR signal of TINO is characterized by three sharp, intense lines due to the nitrogen of one $>N—O\cdot$ moiety and two smaller, broader lines due to the second $>N—O\cdot$ moiety (Figure 5). As incubation time increases, the two broad lines decrease in intensity while the three sharper lines become more resolved. As time proceeds, the signal decreases until complete spectral disappearance after 40 min. These results suggest that in a bis-nitroxide, the two nitroxide moieties react independently with alkyl radicals to give the bis-alkoxyamine and not simultaneously. Figure 6 shows the time profiles of all four nitroxides with alkyl radicals obtained by following the intensity of the mid-field peak of the respective signals due to the nitrogen hyperfine splitting. In particular, Figure 6A shows the intensity profiles of the aromatic nitroxides where the intensity of BC4 increases at first and then starts to decrease for the reasons mentioned earlier, while with MC4 the signal decreases right from the onset of incubation. Similarly, the same is observed for the aliphatic nitroxides (Figure 6B). It is noteworthy to observe that the rate of reaction of these two classes of nitroxides with alkyl radicals is very similar as can be deduced from these time profiles. Figure 6C shows the time profile of the central, narrow line and the first broad line of TINO. In accordance with the results reported in Figure 5, the broad line decreases from the

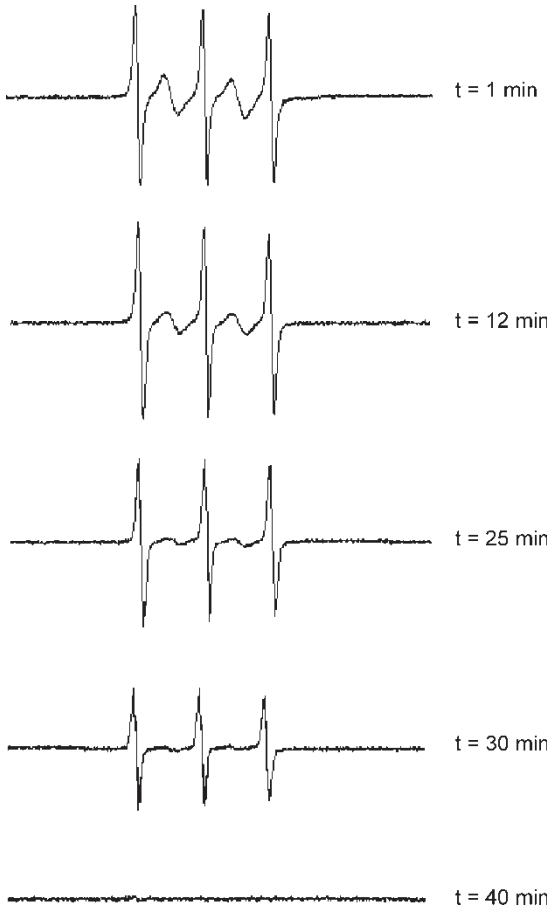


Figure 5. EPR spectral changes of nitroxide TINO in the presence of AMVN and in the absence of oxygen. The nitroxide (200 μM) was incubated with 24 mM AMVN in acetonitrile at 40°C and recordings were continuously taken until signal disappearance.

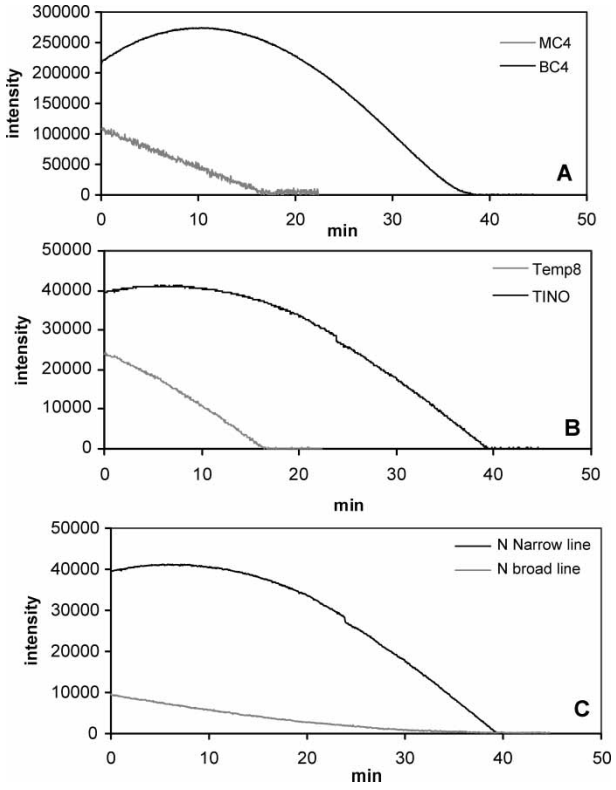


Figure 6. Intensity profiles recorded in time of the mid-field EPR signal peak of aromatic nitroxides (A) and aliphatic nitroxides (B) in the presence of AMVN and in the absence of oxygen. Graph (C) shows the intensity time profiles of the central narrow line and the first broad line of the EPR signal of TINO in the presence of AMVN and in the absence of oxygen. The nitroxides (200 μM) were incubated with 24 mM AMVN in acetonitrile at 40°C and recordings were monitored until signal disappearance.

onset of incubation while the narrow line actually increases slightly at first, remains stable and then slowly starts to decrease until disappearance.

Figure 2B shows the UV spectral changes of MC4 in the presence of AMVN under aerobic conditions, in the presence of peroxyl radicals. The characteristic peak of the nitroxide at 290 nm decreases while the peak at 370 nm remarkably increases. No more spectral changes were observed after 30 min incubation indicating that all the nitroxide present had been transformed. The resulting spectrum obtained is that of the corresponding quinoneimine N-oxide. In fact, aromatic indolinonic nitroxides readily scavenge peroxyl radicals (rate constant between 10^3 and $10^5\,M^{-1}s^{-1}$) [6,13] on the conjugated benzene ring to give the quinoneimine N-oxide as shown is Scheme 1. Similarly, BC4 shows the same spectral pattern as MC4 giving a similar reaction product since the two nitroxides are expected to react in the same way. However, in this case it took roughly 1 h for complete transformation of the bis-nitroxide to the corresponding bis-quinoneimine N-oxide (results not shown).

When the aliphatic nitroxides were incubated with AMVN in the presence of oxygen for the same length of time as the aromatic ones, no appreciable UV–Vis spectral changes were observed suggesting that there is no reaction with peroxyl radicals (results not shown).

These observations were strengthened by the following EPR experiments. When the aromatic mono-nitroxide MC4 was incubated with AMVN without degassing under argon, hence in the presence of oxygen, its spectrum was obviously poorly resolved, showing only three, very poor, broadened lines caused by Heisenberg exchange between molecular oxygen (which is a stable diradical) and the nitroxide. However, as incubation time increased, this signal appreciably decreased in intensity until total disappearance after 20 min (results not shown). This is in accordance with the UV–Vis data obtained (Figure 2B) where the nitroxide upon reaction with peroxyl radicals, was completely transformed into the diamagnetic quinonimine N-oxide after 24 min.

The EPR profile of TEMP8 upon reaction with AMVN in the presence of oxygen is reported in Figure 7 and in this case, interesting results were

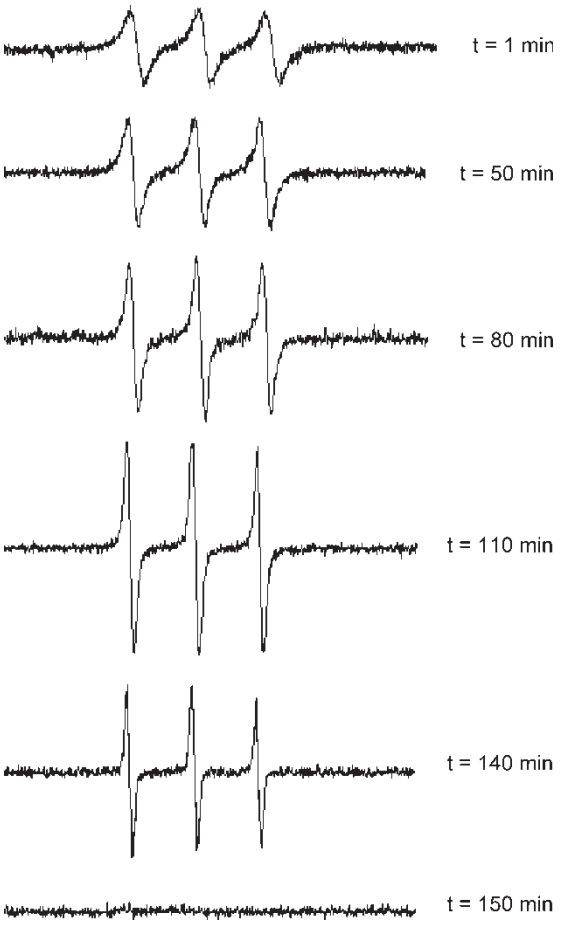


Figure 7. EPR spectral changes of nitroxide TEMP8 in the presence of AMVN and oxygen. The nitroxide (200 μM) was incubated with 24 mM AMVN in acetonitrile at 40°C and recordings were continuously taken until signal disappearance.

obtained. At the onset of incubation, the spectrum consists of three broadened lines, which gradually become sharper, well-defined and increase in intensity as incubation time proceeds. After about 2 h of incubation the spectrum intensity begins to decrease until total disappearance. This unusual behaviour of TEMP8 in the presence of peroxyl radicals may be likely interpreted as follows. At the beginning of incubation, line broadening is observed and this is due to the presence of oxygen in the medium. As alkyl radicals begin to be generated from AMVN these react preferentially with oxygen to give peroxyls. Hence oxygen starts to be consumed and the spectrum of the nitroxide becomes resolved just like when the solution is degassed under argon. The signal does not decrease immediately contrarily to what is observed with MC4. Once all the oxygen in the medium has been consumed, the alkyl radicals begin to be scavenged by the nitroxide to give the alkoxyamine explaining the decrease in intensity and disappearance of the spectrum observed at later incubation times. The formation of the alkoxyamine was confirmed because once the nitroxide had totally reacted with the alkyl radicals, i.e. after spectral disappearance, the temperature of the EPR cavity was increased gradually up to 90°C when the spectrum of the nitroxide was restored (results not shown). This indicates that the alkoxyamine previously formed, starts to break down again at higher temperatures. In fact it is well-known that N-alkoxyamines are thermally unstable and mainly undergo O—C bond cleavage to give the parent nitroxide and because of this, alkoxyamines are now exploited in living free-radical polymerization [11,29]. These EPR results on the aliphatic nitroxide TEMP8 in the presence of peroxyl radicals prompted us to perform additional UV experiments where TEMP8 was reacted with AMVN in the presence of oxygen but the reaction mixture was incubated for 2.5 h and scans were recorded every 15 min. Indeed, after one hour no spectral changes are observed whereas after 2 h the UV spectrum begins to change resembling after the end of incubation, to that observed in Figure 3, i.e. to the alkoxyamine (results not shown). EPR experiments in the presence of oxygen on the bis-nitroxides BC4 and TINO failed since their spectra were very unresolved and too broad.

The antioxidant activities of the nitroxides were then assessed in the oxidation of methyl linoleate micelles in aqueous dispersions induced by AAPH, in order to investigate whether any differences existed amongst the nitroxides in the protection against oxidative damage. This is a convenient model system to evaluate the chemical activity of a compound as an antioxidant against lipid peroxidation, because the free radical-mediated oxidation of methyl linoleate proceeds by a straightforward, well-established mechanism to give conjugated diene hydroperoxides quantitatively [30]. While AAPH is often regarded as water-soluble, in micelles it partitions about 91% into the micellar phase and has a distribution not very different from the 95% observed for linoleic acid [31]. Thus, most of the primary radicals from AAPH probably are produced within or at the surface of the micelle and any lipid-soluble inhibitor, such as the nitroxide compounds considered in this study, would be expected to behave normally by scavenging any radicals generated.

Figure 8 shows the effects of the nitroxides on the extent of lipid peroxidation assessed by monitoring the formation of CD. AAPH induces a time-dependent CD increase in methyl linoleate micelles and all the nitroxides suppress this formation to a different extent. As shown in this figure, a clear inhibition period (lag time) can be observed for the aromatic nitroxides and Trolox (used as reference inhibitor) after which oxidation proceeds at the same rate as in the absence of inhibitor. From these data it is possible to calculate a stoichiometric number n, that is the number of peroxyl radicals scavenged by each antioxidant molecule. Using Trolox as a reference

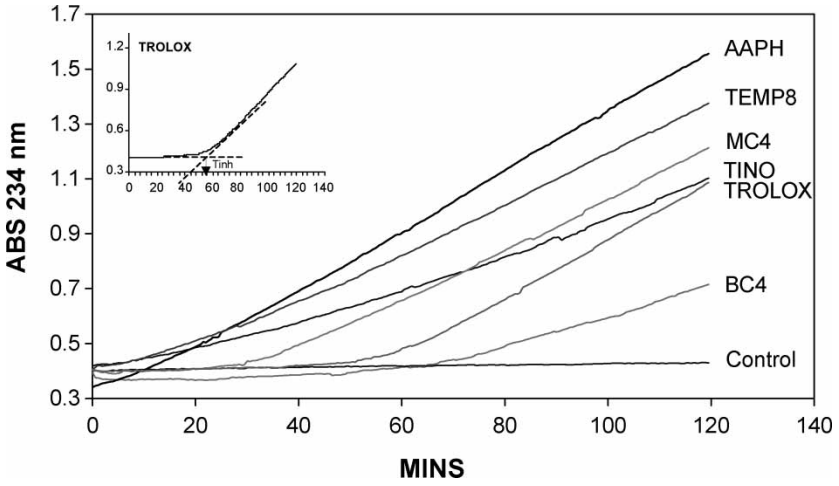


Figure 8. Effect of 1 μM nitroxides and Trolox on the formation of methyl linoleate (1.5 mM) conjugated dienes during peroxidation induced by 1 mM AAPH in a pH 7.4 micellar solution of 0.5 M SDS at 40°C. The control refers to sample lacking AAPH and nitroxides while AAPH indicates the sample without antioxidant addition. The inset shows how the inhibition period was calculated for Trolox: T_{inh} is determined as the point (arrow) of intersection of the two dashed lines.

inhibitor removing two radicals per added molecule [24], it is easy to determine the rate of radical generation (R_i) under our assay conditions from the equation $R_i = n[\text{antioxidant}]/T_{inh}$. Using our experimental R_i value of 5.74×10^{-7}[AAPH] M/s, n for MC4 and BC4 can be deduced, whereas for the aliphatic nitroxides this was not possible since no inhibition time was observed. The stoichiometric number n calculated for MC4 was 1.14 while that for BC4 was 2.17. Furthermore, the concentration of radicals formed from AAPH may be calculated using the following equation:

$$\text{Concentration of radicals formed} = 5.74 \times 10^{-7}[\text{AAPH}]\,\text{M/s} \times T_{inh}.$$

When BC4 was present the concentration of AAPH radicals generated during the inhibition period was 2.17 μM while for MC4 this was 1.13 μM. Therefore it is reasonable that 1 μM BC4 (which traps two peroxy radicals) fully blocks methyl linoleate oxidation for the first 60 min and that 1 μM MC4 fully blocks the oxidation during the first 30 min of the experiment. Even though no clear-cut inhibition period was observed with the aliphatic nitroxides, a continuous and gradual decrease in CD formation was observed during the course of the experiment, which contributes to overall inhibition of lipid peroxidation. In this experimental system, it seems clear that bis-nitroxides inhibit lipid peroxidation better than their corresponding mono-derivatives and that the aromatic nitroxides appear to be better inhibitors than aliphatic ones.

To explore further the antioxidant activity, experiments were also performed on a hydrophilic protein model system consisting of BSA that was oxidized with AAPH in the presence and absence of the nitroxides. The extent of oxidation was determined using DNPH that forms a hydrazone with the carbonyl groups produced during protein oxidation, quantifiable at 370 nm. Figure 9 shows that oxidative modification of BSA during incubation with AAPH is significantly reduced in the presence of the nitroxides only at the higher concentrations tested (100 μM) and that the protection offered by all four nitroxides was similar. Only nitroxide MC4 was able to offer significant protection at the lowest concentration (50 μM).

Discussion

Since the first observations over three decades ago on the protective effects of stable nitroxide compounds

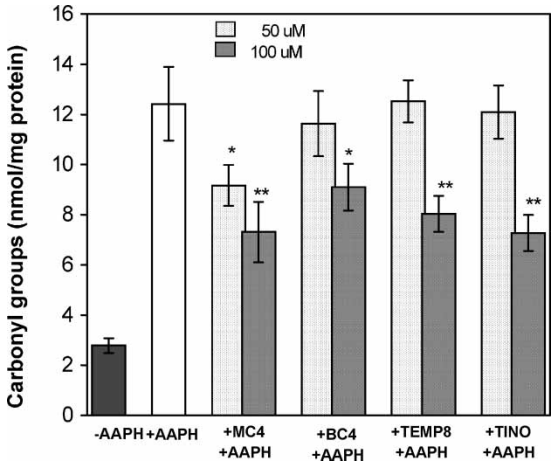


Figure 9. Effect of different concentrations of nitroxides (50 and 100 μM) on protein carbonyl formation in BSA oxidized by exposure to 5 mM AAPH for 1 h at 50°C in 50 mM phosphate buffer, 0.1 mM EDTA, pH 7.4.

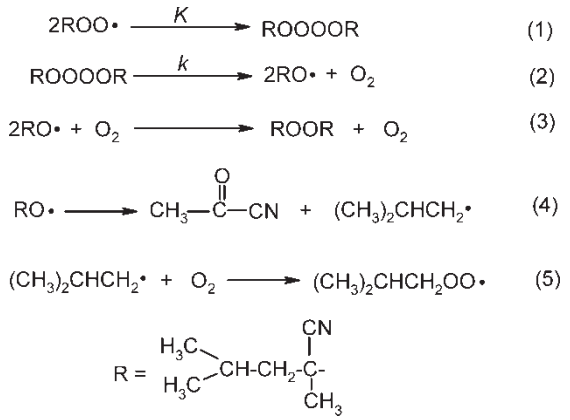
against oxidative damage [32], almost every model system or insult employed so far, for testing the biological effect of nitroxides has demonstrated their antioxidant activity. Not only, these compounds have been used as antioxidants in several other fields such as rubbers [33], polymers [34], paints [35], and even milled wood lignin [36]. The reasons underlying their success is that nitroxides are extremely effective modulators of processes mediated by paramagnetic species (radicals and transition metals) which makes them useful for probing reactions and processes associated with free radicals. All antioxidant studies to date using these compounds have been performed with nitroxides bearing only one $>N—O\cdot$ moiety, while studies on compounds with two nitroxide functions are lacking. For this reason the present work was carried out using both aromatic and aliphatic compounds for comparative purposes, bearing either one or two nitroxide functions. Their radical scavenging behaviour toward alkyl and peroxyl radicals was first investigated based upon the fact that under aerobic conditions, such as those present in most biological tissues, carbon-centred radicals react with oxygen in an almost diffusion-controlled rate to generate peroxyl radicals, the main species involved in chain propagation reactions.

In the present study, the reaction of nitroxides with alkyl radicals generated from AMVN in anaerobic conditions and in homogenous solution, further confirms the well-known reactivity of nitroxides with these radical species: that is, radical–radical coupling at the nitroxide function to give the corresponding alkoxyamine (Scheme 1) [11,12,17]. However, the novelty here is that with bis-nitroxides, the two nitroxide groups appear to scavenge alkyl radicals independent of one another and not simultaneously. In fact, because the two nitroxide groups present in the same molecule react independently, it takes twice as much time for complete spectral transformation (in the case of UV experiments) or EPR signal disappearance of the bis-nitroxides compared to their corresponding mono-nitroxides. This was observed for both aliphatic and aromatic nitroxides and their apparent rate of reaction with these radicals is similar (Figure 6), calculated to be in the order of 10^8–$10^9\,M^{-1}\,s^{-1}$ [17].

However, the reactivity towards peroxyl radicals is still unclear and scarcely investigated, and this is particularly true for aliphatic ones. In 1998 Barton et al. [37] proposed a mechanism whereby the piperidine nitroxide TEMPO reacts with *t*-BuOO· to catalyse the formation of oxygen through a trioxide intermediate. Alternate mechanisms on this reaction were also sought through *ab initio* thermochemical calculations [38]. The formation of an unstable aminotrioxide was again postulated that might decompose yielding oxygen and the corresponding alkoxyamine. Recently, Samuni and Offer [39] have also attempted to address this issue and although some evidence of a reaction taking place was observed, doubts on the true mechanism, if a reaction takes place at all, still remain, especially since compounds deriving from the reactivity of these nitroxides with peroxyls have never been clearly isolated nor identified.

On the contrary, aromatic nitroxides are capable of reacting with peroxyls because of the delocalization of their free valency in the aromatic π system, the rate constants for this reaction being approximately between 10^3 and $10^5\,M^{-1}\,s^{-1}$ [13]. All reaction products and mechanisms have been fully characterized even upon their reaction with other kinds of biologically relevant radicals [11–16] and results now reported here with peroxyls, lend support to the macroscale chemical reactions carried out in earlier years. In fact, in the reaction of aromatic nitroxides with AMVN or AAPH in aerobic conditions, we have direct evidence for the formation of peroxyls that are scavenged on the conjugated benzene ring and the stoichiometric number n calculated for this reaction is roughly 1.14 for MC4 and 2.17 for BC4. This suggests that the reaction of oxygen with the alkyl radicals generated, for example, by AMVN is faster than their coupling at the nitroxide function to give the alkoxyamine because if this latter reaction occurred, we would expect to observe a UV spectrum profile as in Figure 2A.

On the contrary, the reaction of aliphatic nitroxides with peroxyl radicals under the experimental conditions used in this study does not take place. This statement is based principally on the interesting EPR observations reported in the "Results" section. If the mechanism proposed by Offer and Barton was correct [37,39], one would expect spectral decrease right from the onset of the reaction without total disappearance since the nitroxide should be regenerated. However, our findings are in conflict with the mechanism proposed. Further evidence for the non-reactivity of aliphatic nitroxides with peroxyls was from the UV experiments where no spectral changes occur. The peroxyl radicals that are not scavenged by the aliphatic nitroxides may follow different routes (Scheme 2). For example, they may self-react to give a tetroxide (Equation (1)) which then decomposes to alkoxy radicals liberating oxygen (Equation (2))[40]. However, the rate at which these reactions take place (K and k) might depend on the structure of R and to date this question still remains unanswered [40]. Therefore we have no evidence that these specific peroxyl radicals indeed self-react. In the case they do, then the alkoxyl radicals may recombine within the solvent cage to give non-radical products (Equation (3)) or they may escape and undergo β-scission (Equation (4)) to give 2-oxo-propionitrile and the isobutyl radical [40]. This latter radical may react again with oxygen to give the corresponding peroxyl



Scheme 2. The possible fate of the peroxyl radicals deriving from thermal decomposition of AMVN which are represented as ROO·.

(Equation (5)) which may again follow several pathways leading to non-radical products [40]. It is well-known that the fate of peroxyl radicals and products deriving from their self-reaction is rather complex and many pathways may be taken which are beyond the scope of the present discussion. However, it is important to bear in mind that the oxygen liberated in step 2 will be minimal and the rate at which it is formed might be very slow compared to the time course of our reactions. From the results shown here, we believe that the amount of oxygen liberated, if any, is very small otherwise spectral resolution in time followed by spectral decrease and disappearance once oxygen has been consumed, would not be observed.

After having established the radical scavenging activity of the studied nitroxides, their antioxidant efficacy in convenient model systems exposed to oxidative stress was investigated. Lipid peroxidation was first studied because it is a useful and widely recognized marker of the level of oxidative stress, and therefore particularly suitable when undertaking antioxidant studies [41]. From the results reported in Figure 8, it is clear that bis-nitroxides too are capable of effectively inhibiting lipid peroxidation and to a greater extent than their corresponding mono-nitroxides. The fact of bearing two nitroxide moieties which increases the opportunity for scavenging radicals may justify this performance which is particularly evident with the aromatic nitroxides where T_{inh} of MC4 is half that of BC4. The reaction of nitroxides with radicals derived from the azo-initiator prior to their reaction with lipids contributes to the overall inhibition of lipid peroxidation. This implies that in the case of AAPH, the aromatic nitroxides' inhibitory effect includes a chain-terminating step between the inhibitor and the R· radicals, as well as the usual terminating step between the inhibitor and the peroxyl radicals. In the case of the aliphatic nitroxides the inhibition observed which follows a different pattern to the aromatic ones, is most probably ascribed to their reaction with only R· radicals either produced from decomposition of peroxyl radicals (Scheme 2) or from methyl linoleate during its peroxidation or from AAPH itself. In fact, it is worth bearing in mind that the radical scavenging experiments were conducted in acetonitrile while the antioxidant experiments were carried out in buffer. The solubility of oxygen varies up to two orders of magnitude in different solvents, and generally, the solubility in organic solvents is much higher than in water, hence the concentration of oxygen present in acetonitrile is most likely higher than in buffer [42]. This probably explains why aliphatic nitroxides in a buffered system are efficient because the alkyl radicals generated by the azo-initiators have less chance of reacting with oxygen compared to when they are present in an organic solution. They would therefore be expected to be scavenged by the nitroxide before they react with oxygen.

It is now well recognized that proteins are just as susceptible as lipids to free radical attack which results in a wide range of effects including loss of activity, fragmentation, aggregation, oxidation of amino acids [43]. This is thought to be important in several diseases including atherosclerosis. In fact, it has been shown that radical damaged BSA is inefficiently catabolized and accumulates in macrophages thus contributing to the development of this disease [44]. Hence, in order to increase the information on the antioxidant activity of bis-nitroxides, their effect on BSA exposed to AAPH was undertaken. The results show that all the compounds reduced protein carbonyl formation in a concentration-dependent fashion on oxidized BSA but no remarkable differences between the nitroxides were observed, except for nitroxide MC4. This probably signifies that the structure of this nitroxide is the optimum for protecting this particular protein against radical damage.

In conclusion, we demonstrate that: (a) bis-nitroxides are able to scavenge alkyl radicals just as efficiently as mono-nitroxides and that each nitroxide moiety acts independently from the other; (b) aliphatic nitroxides do not appear to react with peroxyl radicals, at least under the experimental conditions used in this study, whereas indolinonic aromatic ones do: their stoichiometric number is 1.14 and 2.17, respectively, for mono- and bis-derivatives; (c) bis-nitroxides are roughly twice more efficient at inhibiting lipid peroxidation compared to their corresponding mono-derivatives; (d) aromatic nitroxides appear to be more effective antioxidants than aliphatic ones, and this is partly explained by their greater ability to scavenge all kinds of radicals involved in oxidative damage of biological systems.

This study provides only comparative information on the relative radical-scavenging and antioxidant activities of mono- and bis-nitroxides, but in spite of this, it gives further insight into

the interesting reactivity of these versatile molecules that are increasingly exploited as antioxidants in several fields.

Acknowledgements

The authors greatly acknowledge the Polytechnic University of the Marche and MIUR for financial support.

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